Journal of Applied Pharmaceutical Science Vol. 14(01), pp 247-260, January, 2024
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2024.155513
ISSN 2231-3354

Evaluation of *in vitro* antidiabetic and antioxidant activity of leaf extracts of *Ecbolium linneanum* kurz.: GC-MS and HR-LCMS-based metabolite profiling and an *in silico* approach

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ARTICLE HISTORY
Received on: 02/08/2023
Accepted on: 10/12/2023
Available Online: 04/01/2024

Key words:
Antidiabetic activity, antioxidant activity, DPPH assay, GC-MS analysis, and ADME profile.

ABSTRACT

The *Ecbolium linneanum* Kurz., plant has been used historically in traditional medicine to treat a wide range of ailments including gout, rheumatism, etc. Therefore, we set out to investigate its anti-diabetic and antioxidant effects. This study was executed by assessing its *in vitro* antidiabetic and antioxidant effects and characterizing the phytochemical composition of the plant material. The antidiabetic action was measured using the α-amylase assay, while antioxidant activity was evaluated using the 2,2-Diphenyl-1-picrylhydrazyl) (DPPH), ferric ion reducing antioxidant power, and hydrogen peroxide assays. The compounds were also put to *in-silico* tests for drug-likeness, absorption, distribution, metabolism, and excretion profile, and binding interactions with α-amylase. In this study, the methanolic leaf extract significantly inhibited α-amylase activity (IC_{50} – 684.94 g/ml). An impressive level of antioxidant activity (87.78% radical scavenging) was measured with the DPPH test. About 57 phytochemicals were discovered in the methanolic leaf extract employing gas chromatography-mass spectroscopy and high-resolution liquid chromatography-mass spectrometry analyses. About 28 compounds were found promising in oral bioavailability and drug-likeness properties in *in-silico* research. A few compounds like rhodotoxin A, pyrophosphite a, and 23-acetoxysoladulcidine demonstrated a high-binding affinity for α-amylase. The findings of this study provide preliminary evidence that leaf extracts of *E. linneanum* may possess anti-diabetic and antioxidant activities. This plant is an excellent source to instigate in researching new natural candidates for antidiabetic therapy.

INTRODUCTION

Diabetes mellitus (DM), which is notable for improper lipid, lipoprotein, and glucose metabolism, has emerged as one of the most common illnesses and is on the rise globally. The Centre for Disease Control and Prevention has recently released the 2022 National Diabetes Statistics Report, as per the report estimates more than 130 million adults are living with diabetes or pre-diabetes in the United States. The prevalence of DM and impaired fasting blood glucose in India was 9.3% and 24.5%, respectively [1]. Among those with DM, 45.8% were aware, 36.1% were on treatment and 15.7% had it under control. It is projected that by 2025 the number of cases of diabetes in India would be 69.9 million with a vast majority still undiagnosed. Given that controlling diabetes can be done with a variety of current treatments, such as insulin injections and oral medications like hypoglycemic pills, the majority of them suffer from a variety of drawbacks, such as being costly or coming with side effects like hepatic toxicity, weight gain, hypoglycemia, gastrointestinal (GI) disturbances, and GI hemorrhage. Because of their accessibility, efficacy, and historical, cultural, and religious preferences, medicinal herbs continue to be a widely used alternative medicine. According to estimates from the WHO, 65%–80% of the world's population lives in underdeveloped nations due to budgetary limitations,

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a lack of access to modern medications, and relies primarily on naturally occurring items for their initial medical care. The global market for medicinal plants expanded from US $23 billion in 2002 to more than US $83 billion in 2008. Only 10% of the world's (2.5 million) species have had the therapeutic potential of their plants and herbs examined since World War I, irrespective of the fact that there has been extensive research done on the problem since that time. This implies that there are still many effective medications that have not yet been explored.

As oxygen is crucial to the survival of life on earth, a small amount of oxygen is converted to numerous free radicals as it is utilized in various living processes. In addition to the above-mentioned pollutants, endogenous ones generated by metabolic processes are also exposed, as are various chemicals and the climate. Reactive oxygen species (ROS) such as superoxide anion (O2•-), hydroxyl (HO•), and peroxyl (ROO•) radicals, as well as reactive nitrogen species (RNS) such as peroxynitrite anion (ONOO-) and nitric oxide (NO•) radical, are present in the exposed oxidants; overall harmful effects occur when these species attack human cells and tissues, leading to cancer [2]. Natural antioxidants in high quantities are frequently found in plants. Antioxidants can stop a chain reaction by scavenging free radicals and preventing oxidation processes [3]. The phytochemicals found in plants, such as phenols, flavanols, carotenoids, and vitamins C and E, can be employed to remove excess radicals from the human body [4].

Ecbolium linneanum, is an indigenous Indian plant that naturally grows along India's eastern coast. Also, it can be found in tropical Asia and Africa. There have been numerous traditional uses for *E. linneanum,* including the treatment of jaundice, menorrhagia, rheumatism, inflammation, etc. Root juice is used to treat premenstrual colic and as an anti-helminthic [5]. The plant is being used for gout, dysuria, and stricture with a leaf decoction. The plant's leaves and roots are used to cure malignancies and used to treat cardiovascular diseases. The extracts from various plant parts, particularly leaf extract, are high in polyphenolic substances such as phenols and flavonoids [6]. Plant components are thought to provide a variety of beneficial health impacts, such as antioxidant properties, which prevent cell damage from ROS and RNS, which can be free radicals, singlet oxygen, and hydroperoxides. Cell damage caused by free radicals is one of the primary causes of aging and several degenerative disorders of aging, such as stress, cancer, cardiovascular disease, immune system degeneration, DM and inflammation, etc. Free radicals can be scavenged or quenched by the body's enzymes, such as glutathione peroxidase and superoxide dismutase, to defend the body from negative impacts. These enzymes might be assisted in doing so by phytochemicals that exist in plants. Numerous assays, such as the DPPH, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid, ferric ion reducing antioxidant power (FRAP), and CUPRAC tests, have been used to evaluate the antioxidant capabilities of plant extracts. These methods have shown varying results among the examined plants and laboratories.

The leaves of *E. linneanum* have been demonstrated to be a possible hypoglycemic source in an earlier investigation [7]. Therefore, we broadened our study to include *in vitro* assays on target enzyme activity, such as α-amylase, to assess the antidiabetic potentials of leaf extracts. *In vitro*, testing was used to ascertain the extracts' anti-oxidant properties. Phytocompounds identified by gas chromatography-mass spectroscopy (GC-MS) and high-resolution liquid chromatography-mass spectrometry (HR-LCMS) investigations and were further analyzed virtually for binding interactions with α-amylase using the Protein Data Bank (PDB) crystal structure (1b2y).

MATERIALS AND METHODS

Chemicals

Folin ciocalteu (FC) reagent, gallic acid, quercetin, 2,2-Diphenyl-1-picrylhydrazyl) (DPPH), ascorbic acid (ABA), hydrogen peroxide (H_2O_2), phosphate buffer, potassium ferricyanide, trichloroacetic acid, ferric chloride ($FeCl_3$), etc., were purchased from Merck India, Mumbai. The α-amylase was purchased from Sigma-Aldrich, India. All the solvents and reagents used were of analytical grade and obtained from Merck India, Mumbai.

Plant material

In the first week of September 2018, *E. linneanum* leaves were acquired as raw material from the wastelands of Guntur, Andhra Pradesh, India. The collected leaves were recognized by Dr. Madhav Shetty, a professor in the Department of Botany at Sri Venkateshwara University in Tirupati, Andhra Pradesh, India. The leaves were scrubbed until there was no longer any sign of foreign matter in distilled water, detergent, and tap water. To air dry, the leaves were kept at room temperature in the shade. The dried leaves were freed from the moisture and processed into a powder using a sterile electrical blender. The powdered substance was then kept in an airtight container for later use [8].

Extraction

A Soxhlet apparatus was used to get the crude leaf extracts of petroleum ether (PEEL), ethyl acetate (EAEL), and methanolic (MEEL) from 180 g of coarse powder throughout 72 hours. By utilizing a rotary evaporator, the crude filtrate was concentrated. The resultant extracts were kept in a refrigerator at 4ºC for later use [8].

Phytochemical analysis

To determine the presence of a variety of secondary metabolites in leaf extracts qualitative phytochemical testing was conducted. The earlier investigations presented that the MEEL was comprised of rich amounts phytochemicals, such as alkaloids, glycosides, phenols, and flavonoids [8].

Total phenolic content (TPC)

The FC colorimetric method [9] was used to assess the TPC of *E. linneanum* leaf extracts. About 100 µl of the diluted extracts, 500 µl of the FC reagent, and 400 µl of 7.5% (*w*/*v*) saturated aq. Na_2CO_3 were combined thoroughly. Then, it was homogenized and incubated for 30 minutes at 40ºC. After incubation, each sample's absorbance was measured at 765 nm using methanol as the blank. The calibration curve approach was used to ascertain the TPC using gallic acid as the standard. Each test was run in triplicate, and the results were expressed as gallic acid equivalents (GAE) in mg per g of dry extract (mg GAE/g) [9].

Total flavonoid content (TFC)

TFC of *E. linneanum* leaf extracts was assessed by the aluminum chloride ($AlCl_3$) colorimetric technique [10]. In this method, 125 µl of sodium nitrite solution (5%), 250 µl of each solvent extract, and 1,250 µl of distilled water were combined. The contents were then let to stand for 6 minutes at room temperature. 150 µl of 10% $AlCl_3$ solution was then added, and the mixture was left to stand for another 6 minutes. Following that, 275 µl of distilled water was used to dissolve 500 µl of sodium hydroxide solution (4%). The mixture was completely combined and left to stand at room temperature for 30 minutes. At 510 nm, the absorbance was measured in comparison to a reagent blank that contained methanol. The measurement of total antioxidant capacity was performed using quercetin as a reference (QE) per mg of dry extract (µg QE/mg) [11].

***In vitro* antidiabetic activity**

The *in vitro* antidiabetic activity was assessed by performing **α-amylase** inhibitory assay. In which the sample solution (100 µg/ml) of all the solvent extracts was made by using phosphate buffer (pH-6.8). 500 µl of test solution was added with α-amylase (0.5 mg/ml) solution. The resulting solution was incubated for 10 minutes at 25ºC [12]. Further procedure was followed to estimate the inhibitory effect on enzyme activity taking acarbose as a reference standard. The inhibitory effect on the α-amylase activity of solvent extracts was statistically expressed in terms of their IC_{50} values [13].

Antioxidant activity

The solvent extracts were subjected to screen for their possible antioxidant capabilities by DPPH, H_2O_2, and FRAP methods.

DPPH free radical-scavenging assay

The free radical scavenging assay of the extracts (PEEL, EAEL, and MEEL) utilizes the stable free radical of DPPH according to Yeo and Shahidi [14] DPPH methanolic solution (1 ml, 0.2 mM) was added to the extract solution (1 ml, 2.5–100 µg/ml). The mixture was vortexed thoroughly and incubated for 30 minutes at room temperature. The absorbance was measured at 517 nm with methanol as a blank. ABA as a positive control, the DPPH activity was measured by using the below equation:

Radical scavenging activity (% inhibition) = (Abs. blank–Abs. sample)/Abs. blank × 100

IC_{50} value of solvent extract was calculated from the regression line [14].

H_2O_2 radical scavenging assay

The H_2O_2 scavenging assay was carried out by following the standard procedure described by Ruch *et al*. [15]. A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH—7.4). The three extracts (PEEL, EAEL, and MEEL) at different concentrations (1 ml, 2.5–100 µg/ml) in 3.4 ml phosphate buffer were added to 0.6 ml of H_2O_2 solution (43 mM). The absorbance of the mixture was measured at 230 nm [16].

H_2O_2 scavenging activity (% inhibition) = (Abs. blank–Abs. sample)/Abs. blank × 100

The IC_{50} value of solvent extracts was measured from the regression line of the % of remaining H_2O_2 radical with the sample concentration.

FRAP assay

The method described by Gohari *et al*. [17] was used to measure the reducing power of the PEEL, EAEL, and MEEL. 1 ml of test sample solution at various concentrations (2.5–100 µg/ml) was combined with 2.5 ml of potassium ferricyanide (1% *w/v*), 2.5 ml of phosphate buffer (0.2 M, pH–6.6) and 2.5 ml of 10% trichloroacetic acid, which was then incubated for 20 minutes at 50ºC. After the mixture was centrifuged for 10 minutes at 3,000 rpm, 2.5 ml of the upper layer was removed and combined with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The calibration curve approach was used to determine the % inhibition using the standard ABA (10 g/ml). A spectrophotometer was used to measure each absorbance in triplicate at 700 nm [17].

Ferric reducing power (% inhibition) = (Abs. blank–Abs. sample)/Abs. blank × 100

The IC_{50} of solvent extracts was analyzed from the regression line of the % of remaining ferric ion radical at the test concentration of extract.

GC-MS and HR-LCMS study

Conventional methods for identifying bioactive phytoconstituents require a variety of procedures, including extraction, chromatographic separation, and spectroscopic characterization. Nevertheless, despite substantial time and effort, most researchers only attempt to characterize a small number of known phytochemicals due to the lack of appropriate phytochemical standards. To identify and simplify efforts to understand their action on the target, high throughput, and high-resolution approaches must be used to reveal the complex chemistry of bioactive crude extracts. Extensive research has been conducted to better understand the medicinal applications of the *Ecbolium* genus. However, despite its usage as a replacement constituent for *E. linneanum*, efforts toward knowing the chemistry of the plant have remained significantly low. The preliminary phytochemical study confirmed that the MEEL comprised diverse classes of phytoconstituents like alkaloids, flavonoids, phenols, saponins, etc. Besides, it displayed significant activity in *in vitro* and *in vivo* studies. Thus, as per the findings of qualitative phytochemical tests and biological efficacies, the MEEL was subjected to GC-MS and HR-LC-electrospray ionization (ESI)-MS/MS analysis to determine the presence of diverse phytochemicals [18].

GC-MS analysis

All the probable phytochemicals that are separated from the sample will be detected as a spectral emissary by this approach. After injecting the sample into the GC-MS device's port, the sample is vaporized and then separated using an analyzer. Each component is generating a clean, identifiable peak, which was recorded digitally on a graph. The analysis was performed at CSIR-Indian Institute of Chemical Technology,

Habsiguda, Hyderabad. The data was recorded on a combined gas chromatogram system (Agilent GC-MS5977B) and mass spectrometer, fitted with an HP-5 MS fused silica column (5% phenyl methyl siloxane 30.0 m × 250 µM, film thickness 0.25 µM), interfaced with 5675C Inert MSD with Triple-Axis detector.

HR-LCMS analysis

In HR-LCMS analysis, the phytoconstituents were profiled based on the retention time (Rt), *m/z* values, NIST library hits, and metabolite class. The metabolite analysis in MEEL was performed by HR-LCMS, UHPLC-PDA detector-ESI-QTOP-MS (Agilent Technologies, USA). With the aid of mass spectra and distinctive mass fragmentation patterns, compounds were discovered. For the chromatographic separation, Hypersil GOLD C18 (2.1 × 100 mm 3-µ) column was used with a gradient solvent system, (A) water with 0.1% formic acid and (B) 90 % acetonitrile with 10% water + 0.1% formic acid, A 95% B 5% for 1 minute, B 100% for 2–30 minutes, A 95% B 5%, for 30–35 minutes at 0.3 ml/minute flow rate with pressure maintained at 1,200 bar. The mass spectral data were acquired in both ESI-positive and negative ionization modes. MS was acquired over the *m/z* range of 100–1,200 at a mass resolution of 22,000 full-width half at maximum [19].

Table 1. TPC and TFC.

Extract	TPC (mg GAE/g dried extract)	TFC (mg QE/g dried extract)
PEEL	12.76 ± 0.19	4.21 ± 0.23
EAEL	71.34 ± 0.23	15.54 ± 0.18
MEEL	106.11 ± 0.14	20.83 ± 0.15

In silico absorption, distribution, metabolism, and excretion (ADME) study

The ADME properties of discovered metabolites in GC-MS and HR-LCMS were assessed using an online tool (SwissADME), to choose the most promising compounds with minimal risk of drug attrition in the later studies. The metabolites with the most reliable ADME properties have been taken into consideration for docking study [20].

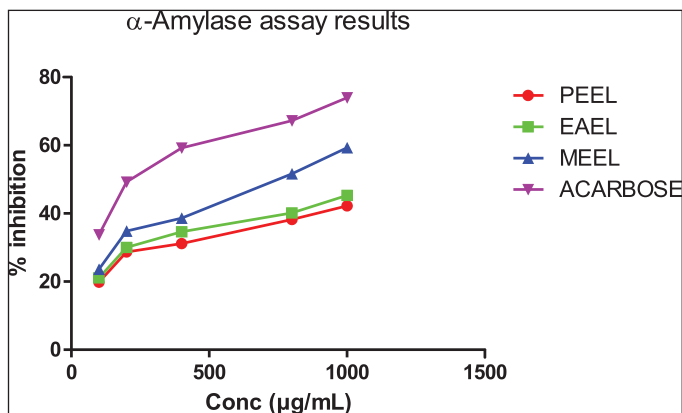

Figure 1. % Inhibition α-amylase activity of leaf extracts and Acarbose. PEEL—Petroleum ether extract; EAEL—Ethyl acetate extract; MEEL—Methanol extract.

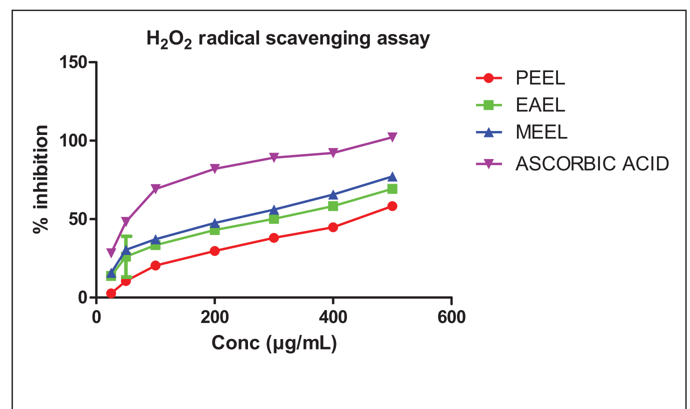

Figure 3. % Inhibition of H_2O_2 radical scavenging of plant extracts and ABA. PEEL—Petroleum ether extract; EAEL—Ethyl acetate extract; MEEL—Methanol extract.

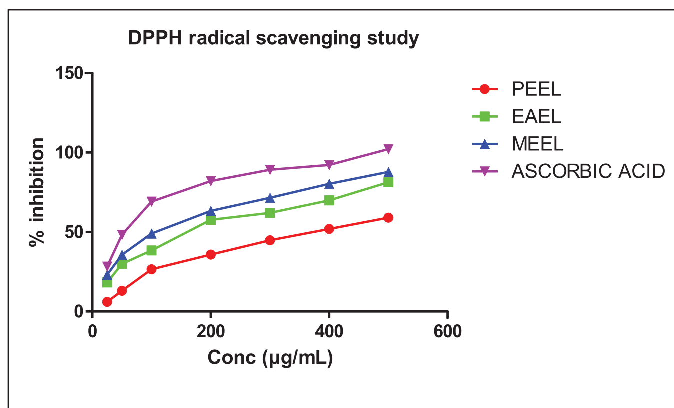

Figure 2. % Inhibition of DPPH radical scavenging of leaf extracts and ABA. PEEL—Petroleum ether extract; EAEL—Ethyl acetate extract; MEEL—Methanol extract.

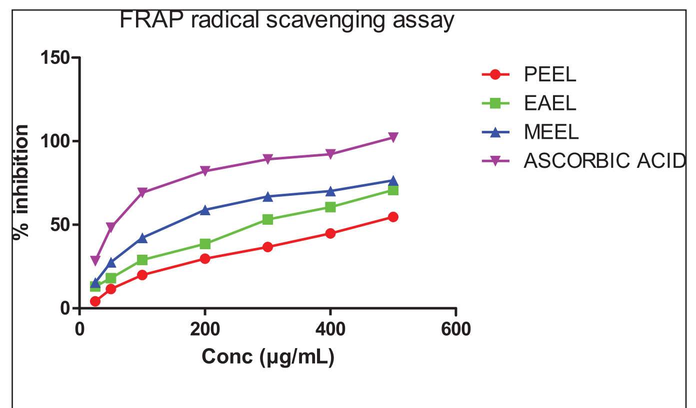

Figure 4. % Inhibition by FRAP of plant extracts compared with ABA. PEEL—Petroleum ether extract; EAEL—Ethyl acetate extract; MEEL—Methanol extract.

Docking study

The X-ray crystal structure of the α-amylase (PDB ID: 1b2y) [21] protein co-crystallized with acarbose was retrieved from a protein data bank (rcsb.com/pdb database). The protein was refined by eliminating water molecules, adding polar hydrogen. The co-crystal ligand was extracted using the Discovery studio visualizer 2021 program and saved in pdb format. The protein was checked for any missing amino acid residues, and the Ramachandran plot was used to check for any structural problems. The created protein file in pdb format was converted to pdbqt format using the macromolecule option in the Autodock tool of the PyRx virtual screening application 0.8. The target compounds 2D structures were drawn and their energy was minimized in 3D structure using ChemDraw software tools and saved as pdb files. The saved .pdb files were subjected to energy minimization (force field-off), and then generated conformers (AutoDock pdbqt files) using the open babel tab in PyRx software. The macromolecule (protein pdbqt file) and ligands were chosen using Vina Wizard to do the docking study (Autodock pdbqt files) by drawing a grid box around the area where the co-crystal ligand exhibits iterations with amino acids, the active site of the protein was defined to dock ligands to proteins. The potential compounds with a high binding affinity against the target protein were identified as the ligands with the lowest binding energies. The binding interactions were visualized using the Discovery Studio Visualizer 2021 program.

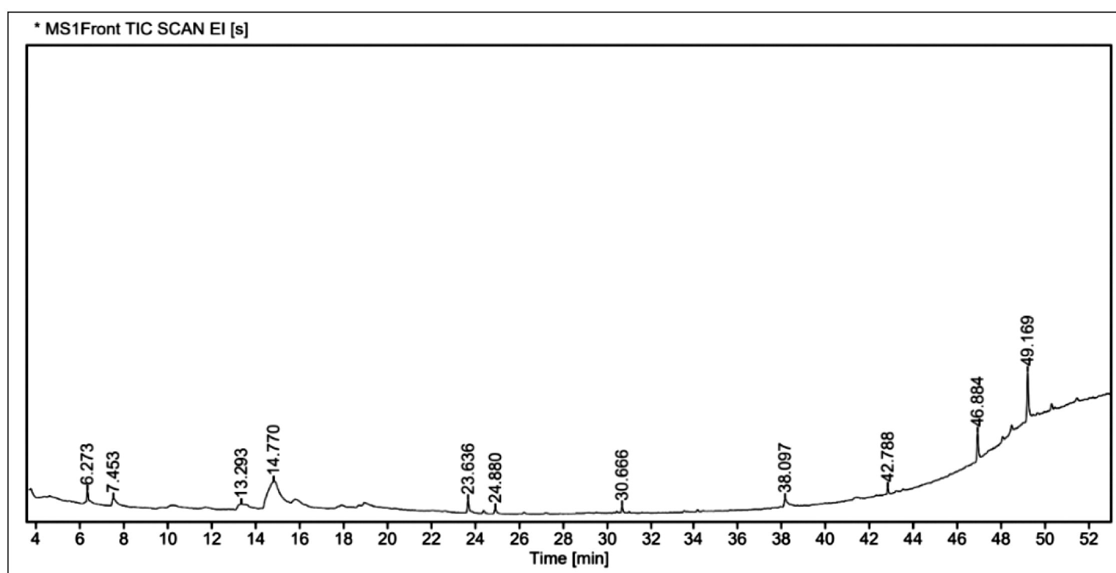

Figure 5. GC chromatogram of MEEL.

Table 2. List of phytochemicals identified by GC-MS analysis in MEEL.

Compound	Mol. Formula	M.W (g/mol)	Rt (min)	Area (%)	PubChem CID	Biological activity
Undecanal,2-methyl	$C_{12}H_{24}O$	184.32	6.275	4.85	61031	Fragrance agent [23]
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144.12	7.457	4.49	119838	Antioxidants [24]
2-Pentylcyclo hexane-1,4-diol	$C_{11}H_{22}O_2$	186.29	13.289	8.08	534646	None
l-Gala-l-ido-octose	$C_8H_{16}O_8$	240.21	14.771	46.19	219659	Used in dementia [25]
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5	23.635	4.05	5366244	Antioxidants [26]
Phytol	$C_{20}H_{40}O$	296.5	30.667	1.64	5280435	Antioxidant and fragrance agents [27]
Hexadecanoic acid,1-(hydroxyl methyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568.9	38.094	5.33	10686914	α-reductase inhibitor and antioxidant [28]
Ethyliso-allocholate	$C_{26}H_{44}O_5$	436.633	42.788	0.79	6452096	Dihydropteroate synthase inhibitor [29]
Peiminine	$C_{27}H_{43}NO_3$	429.635	46.889	6.70	167691	Anti-inflammatory agent [30]
γ-Sitosterol	$C_{28}H_{50}O$	402.39		17.34	457801	Hypoglycemic [22]

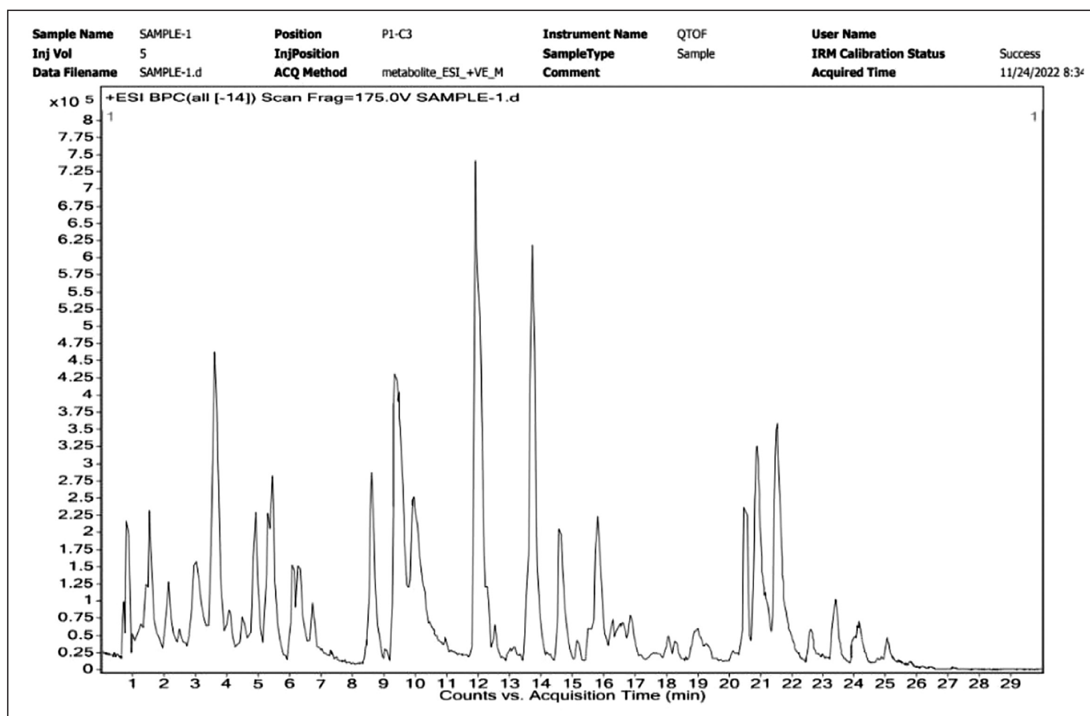

Figure 6. LC chromatogram (ESI positive mode) of MEEL.

Table 3. List of phytochemicals identified by HR-LCMS analysis in MEEL.

S.no	Compound label	Rt	Mass	Formula	DB Diff (ppm)	Hits (DB)
1	(+/−)-3-[(2-methyl 3- furl)thiol]-2-butanone	0.777	184.0572	$C_9H_{12}O_2S$	−7.84	1
2	8-Hydroxy-2- chlorodibenzofuran	0.833	218.0134	$C_{12}H_7ClO_2$	0.19	2
3	Sinapoyl malate	1.363	340.0779	$C_{15}H_{16}O_9$	4.44	6
4	Nonyl octanoate	1.437	270.2529	$C_{17}H_{34}O_2$	11.2	10
5	2,7-Anhydro-α-*N*- acetylneuraminic acid	1.44	291.094	$C_{11}H_{17}NO_8$	4.87	8
6	2′-Deoxyadenosine	1.461	251.1019	$C_{10}H_{13}N_5O_3$	−0.12	9
7	Fenapanil	1.575	253.154	$C_{16}H_{19}N_3$	15.34	1
8	Saxitoxin	1.577	299.1359	$C_{10}H_{17}N_7O_4$	−5.53	2
9	Prolyl-arginine	1.584	271.1646	$C_{11}H_{21}N_5O_3$	−0.43	7
10	*N*-Arylglycine methyl ester	2.162	143.0577	$C_6H_9NO_3$	3.98	5
11	Melochinone	2.426	331.1637	$C_{22}H_{21}NO_2$	−19.45	1
12	5-Methoxytryptophol	2.991	191.0934	$C_{11}H_{13}NO_2$	6.43	7
13	1α-*O*- Methylquassin	3.452	404.2154	$C_{23}H_{32}O_6$	10.99	6
14	Ricinine	3.852	164.0578	$C_8H_8N_2O_2$	4.69	5
15	*N*-n-Hexanoylglycine methyl ester	3.898	187.122	$C_9H_{17}NO_3$	−6.1	7
16	5-(3-Hydroxy-4- acetoxybut-1-ynyl)-2,2′bithiophene	4.633	292.021	$C_{14}H_{12}O_3S_2$	6.16	2
17	2,4,6-Trihydroxytoluene	4.936	140.0466	$C_7H_8O_3$	5.16	10
18	Erinapyrone C	5.432	186.0539	$C_8H_{10}O_5$	−5.8	3
19	2′- Aminoacetophenone	5.433	135.0677	C_8H_9NO	5.43	10
20	Isoscopoletin	6.116	192.0412	$C_{10}H_8O_4$	5.61	10
21	Fraxidin	6.699	222.0521	$C_{11}H_{10}O_5$	3.21	10
22	Perlolyrine	7.07	264.0886	$C_{16}H_{12}N_2O_2$	4.71	9
23	7-Dehydrologanin tetraacetate	8.517	556.1795	$C_{25}H_{32}O_{14}$	−0.45	10
24	Torachrysone 8-β-gentiobioside	8.603	570.1955	$C_{26}H_{34}O_{14}$	−1.2	7

Continued

S.no	Compound label	Rt	Mass	Formula	DB Diff (ppm)	Hits (DB)
25	2-(3-Phenylpropyl) tetrahydrofuran	9.057	190.1347	$C_{13}H_{18}O$	5.82	9
26	Celereoside	9.1	424.138	$C_{20}H_{24}O_{10}$	−2.48	10
27	Procyanidin B7	9.663	578.1411	$C_{30}H_{26}O_{12}$	2.27	10
28	Morindone	9.712	270.0523	$C_{15}H_{10}O_5$	1.98	10
29	C16 Sphinganine	11.90	273.2664	$C_{16}H_{35}NO_2$	1.34	1
30	2″,3″-Di-O-p- coumaroylafzelin	12.00	724.1765	$C_{39}H_{32}O_{14}$	3.69	1
31	Phytosphingosine	12.37	317.2916	$C_{18}H_{39}NO_3$	4.43	1
32	Palmitic amide	12.61	255.2553	$C_{16}H_{33}NO$	3.77	1
33	6- Hydroxypentadecanedioic acid	12.83	288.1946	$C_{15}H_{28}O_5$	−3.08	4
34	(-)-Solenopsin A	13.59	253.2762	$C_{17}H_{35}N$	2.87	1
35	Nigakilactone B	13.66	392.2206	$C_{22}H_{32}O_6$	−1.74	10
36	(5α,8β,9β)-5,9-Epoxy-3, 6-megastigmadien-8-ol	14.22	208.1464	$C_{13}H_{20}O_2$	−0.58	10
37	Gingerglycolipid A	14.36	676.3642	$C_{33}H_{56}O_{14}$	4.1	6
38	12-Hydroxy-8,10-octadecadienoic acid	14.59	296.2361	$C_{18}H_{32}O_3$	−3.28	10
39	Lasiodine A	14.65	698.3463	$C_{39}H_{49}N_5O_7$	1,456.11	2
40	Flavidulol C	15.85	514.3118	$C_{34}H_{42}O_4$	−6.88	4
41	LysoPE(20:0/0:0)	15.94	509.3564	$C_{25}H_{52}NO_7P$	−16.23	2
42	Retapamulin	16.09	517.3163	$C_{30}H_{47}NO_4S$	12.06	1
43	Rhodexin A	16.15	536.2943	$C_{29}H_{44}O_9$	7.94	4
44	*trans*-heptaprenyl diphosphate	16.29	654.3805	$C_{35}H_{60}O_7P_2$	1.38	4
45	Linoleoyl ethanolamide	18.08	323.2813	$C_{20}H_{37}NO_2$	3.42	2
46	4,4′-Diapo-zeta-carotene	18.34	404.342	$C_{30}H_{44}$	5.79	2
47	23-Acetoxysoladulcidine	18.83	473.3488	$C_{29}H_{47}NO_4$	3.68	1
48	Oxidized dinoflagellate luciferin	20.19	602.2745	$C_{33}H_{38}N_4O_7$	−0.77	3
49	Neomycin B	20.51	614.3146	$C_{23}H_{46}N_6O_{13}$	−3.75	2
50	Pheophorbide a	20.96	592.267	$C_{35}H_{36}N_4O_5$	2.72	2
51	Ganoderic acid F	21.34	570.2848	$C_{32}H_{42}O_9$	−3.33	2
52	Pyropheophorbide a	21.72	534.2614	$C_{33}H_{34}N_4O_3$	3.16	10
53	O-Methylsomniferine	21.84	622.2773	$C_{37}H_{38}N_2O_7$	−15.11	2
54	Ganosporelactone A	22.12	512.2791	$C_{30}H_{40}O_7$	−3.4	10
55	Antimycin A1	23.33	548.2766	$C_{28}H_{40}N_2O_9$	−5.78	1
56	7b-Hydroxy-3-oxo-5b- cholanoic acid	23.37	390.2759	$C_{24}H_{38}O_4$	2.81	10
57	D8′-Merulinic acid A	23.41	390.2753	$C_{24}H_{38}O_4$	4.5	10

RESULTS AND DISCUSSION

Phytochemical analysis

The initial phytochemical analysis showed that each solvent leaf extract contained a distinctive variety of phytoconstituents. Saponins and tannins were the primary components of all three solvent extracts. However, the ratio of other phytoconstituents varied [7].

TPC and TFC

In Table 1, the amounts of TPC and TFC were shown as mg/g. When compared to the PEEL and EAEL, the MEEL comprised the highest levels of TPC and TFC. The phytochemical content of MEEL was thought to be high, so the plant extract was tested for its ability to combat diabetes and free radical damage [9,11].

In vitro antidiabetic activity

The α-amylase inhibiting activity of leaf extracts (PEEL, EAEL, and MEEL) was determined *in vitro* using the standard method. In this investigation, a dose-dependent increase in α-amylase inhibitory activity was observed. The extract demonstrated 21.60% ± 0.2354 inhibition at a concentration of 100 µg/ml, and 59.31% ± 0.352 at a concentration of 1,000 µg/ml (Fig. 1). The extract revealed an IC_{50} of 684.94 ± 3.96 µg/ml, while acarbose's IC_{50} was 322.50 ± 4.5 µg/ml [13].

Antioxidant activity

The antioxidant capabilities of three solvent extracts determined by DPPH, H_2O_2, and FRAP assays were presented in the following,

DPPH radical scavenging assay

MEEL inhibited (87.78% ± 0.16 ***) the DPPH radical significantly more than the other two extracts. The % radical scavenging inhibition of MEEL was nearly alike to that of ABA (102.19% ± 0.13%) (Fig. 2).

H_2O_2 radical scavenging assay

The H_2O_2 radical scavenging assay was tested at 25–500 µg/ml of three solvent extracts, in which MEEL exhibited the highest (77.22% ± 0.43%) radical scavenging activity at 500 µg/ml. The PEEL and EAEL extracts were moderately potent at 500 µg/ml (Fig. 3).

Table 4. Chemical classes of phytochemicals identified by GC-MS and HR-LCMS analysis.

S.no	Compound Label	Category	Reported activity
1	(-)-Solenopsin A	Alkaloids	Insecticidal [31]
2	23- Acetoxysoladulcidine		
3	5-Methoxytryptophol		Anti-anxiety agent [32]
4	O-Methylsomniferine		Flavouring agent [33]
5	Perlolyrine		PDE5 inhibitor [34]
6	Ricinine		Lubricant [35]
7	Melochinone		Clotting agent [36]
8	(+/-)-3-[(2-methyl 3- furl) thiol]-2-butanone	Flavonoids	
9	(5α,8β,9β)-5,9-Epoxy-3,6-megastigmadien-8-ol		
10	2'- Aminoacetophenone		
11	2-(3 Phenylpropyl) tetrahydrofuran		Flavouring agent [37–43]
12	2",3"-Di-O-*p*-coumaroylafzelin		
13	Nonyl octanoate		
14	D8'-Merulinic acid A		
15	Erinapyrone C		
16	Flavidulol C		
17	4,4'-Diapo-zeta- carotene	Lipids	
18	Linoleoyl ethanolamide		Fatty acid amide hydrolase) inhibitor[44]
19	LysoPE(20:0/0:0)		
20	Phytosphingosine		Treating skin diseases [45]
21	Gingerglycolipid A		Antioxidant and anti-inflammatory agent [46]
22	Palmitic amide	Fatty acids	
23	12-Hydroxy-8,10-octadecadienoic acid		Antioxidant activity[30]
24	6-Hydroxypentadecanedioic acid		
25	Celereoside	Steriod	[47]
26	7b-Hydroxy-3-oxo-5b-cholanoic acid		
27	Ganoderic acid F		5-α-reductase inhibition [48]
28	N-Arylglycine methyl ester		
29	Rhodexin A		Cytotoxic agent [49]
30	1α-O- Methylquassin		
31	Neomycin B	Glycoside	Antibiotics [50,51]
32	Nigakilactone B		Antiviral [52]
33	N-n-Hexanoylglycine methyl ester		Anticancer [53]
34	Isoscopoletin		Antioxidant and anti-asthmatic
35	Torachrysone 8-β-gentiobioside		
36	2'-Deoxyadenosine	Purine nucleoside	Antiviral agent
37	7-Dehydrologanin tetraacetate	Terpene	[54]
38	Procyanidin B7	Tannins	Anti-inflammatory
39	Antimycin A1	Macrocyclic	Insecticide
40	Fraxidin	Coumarin	Antibiotic
41	Ganosporelactone A	Steroid	Antihypertensive
42	Lasiodine A	Alkaloid	
43	Morindone	Anthraquinone	Anticancer agent
44	Prolyl-Arginine	Dipeptide	
45	8-Hydroxy-2-chlorodibenzofuran	-	
46	5-(3-Hydroxy-4-acetoxybut-1-ynyl)-2,2'bithiophene	-	
48	Fenapanil	-	Fungicide [55]
49	2,4,6-Trihydroxytoluene	Phenols	
50	2,7-Anhydro-α-*N*- acetyl neuraminic acid	Carbohydrate	
51	*trans*-heptaprenyl diphosphate	-	
52	Oxidized dinoflagellate luciferin	Protein	
53	Pheophorbide a	Porphyrins	Antioxidant and anti-inflammatory, anticancer agent [56]
54	Pyropheophorbide a		Antioxidant [57]
55	Retapamulin	Tricyclic diterpenoid	Topical antibiotic [58]
56	Saxitoxin	Alkaloid	Neurotoxin [59]
57	Sinapoyl malate	Coumaric acid	Sun screen agent [60]

Table 5. ADME profile of identified phytochemicals as per *in-silico* study.

S. no.	Molecule	TPSA	Consensus Log P	Ali Log S	GI abs.	BBB per.	Pgp sub.	log Kp (cm/second)
1	2-(3-Phenylpropyl) tetrahydrofuran	9.23	3.24	−3.17	High	Yes	No	−5.12
2	2,4,6-Trihydroxy toluene	60.69	0.96	−2.09	High	Yes	No	−6.29
3	2,7-Anhydro-α-*N*-acetylneuraminic acid	145.55	−1.93	0.53	Low	No	Yes	−10.22
4	2′-Aminoacetophenone	43.09	1.43	−2.15	High	Yes	No	−5.97
5	4,4′-Diapo-zeta-carotene	103.01	3.27	−5.25	High	No	No	−5.66
6	5-(3-Hydroxy-4-acetoxybut-1-inly)-2,2′-bithiophene	45.25	1.85	−2.65	High	Yes	No	−6.00
7	5-Methoxytryptophol	60.44	4.43	−5.54	High	Yes	No	−5.53
8	7b-Hydroxy-3-oxo-5b- cholanoic acid	63.32	0.72	−2.14	High	Yes	No	−6.28
9	Anthranilic acid	66.48	4.39	−6.97	High	Yes	Yes	−4.02
10	C16 Sphinganine	41.61	2.79	−3.48	High	Yes	No	−5.76
11	D8′-Merulinic acid A	68.9	1.49	−2.55	High	Yes	No	−6.59
12	Fenapanil	59.67	1.51	−2.24	High	Yes	No	−6.49
13	Fraxidin	103.78	−0.57	0.6	High	No	No	−9.19
14	Isoscopoletin	94.83	2.03	−4.94	High	No	No	−5.63
15	Methyldopa	62.05	2.55	−3.17	High	Yes	Yes	−6.33
16	Morindone	86.71	3.67	−6.19	High	No	Yes	−4.94
17	Nonyl octanoate	85.61	0.45	−1.29	High	No	Yes	−7.81
18	Perlolyrine	55.02	0.5	−0.72	High	No	No	−7.29
19	Pheophorbide A	139.59	0.88	−3.43	High	No	No	−7.73
20	Rhodexin A	145.91	1.61	−3.59	Low	No	Yes	−8.91
21	23-Acetoxy soladulcidine	67.79	4.33	−6.16	High	Yes	Yes	−5.63

Note: Lipinski violations - No.

FRAP assay

The FRAP assay was tested at 25–500 µg/ml of three solvent extracts. In this study, the MEEL exhibited 76.56% ± 0.27% of inhibition at 500 µg/ml, which was a similar impact observed in H_2O_2 radical scavenging assay. The PEEL and EAEL extracts were found less potent at 500 µg/ml (Fig. 4).

GC-MS and HR-LCMS study

GC-MS analysis

Based on the *m/z* and Rt of each fraction from the column, the plant compound was searched in the NIST library for the likely components there in the leaf extract (Fig. 5). The GC-MS reports stated the existence of bioactive metabolites in varied proportions such as l-Gala-l-ido-octose (46.19%), γ-Sitosterol (17.34%), Peiminine (6.7%), and 4H-Pyran-4-one, 2, 3-dihydro-3,5-dihydroxy-6-methyl (4.49%) etc (Table 2). The majority of compounds have been reported to poses antioxidant, anti-inflammatory, and hypoglycemic properties.

HR-LCMS analysis

The *m/z* values were in the range of 135 to 724 for the majority of separated compounds in MEEL. The HR-LCMS study could provide information on the distribution of various polar to non-polar phytochemicals. ESI positive and negative ion modes were applied to identify all types of small molecules. The representative base peak LC 8chromatogram of MEEL was depicted in Figure 6.

Table 3 which summarized the tentative metabolites characterized from MEEL includes their Rt, experimental *m/z*, mass, proposed metabolites, molecular formula, etc. The HR-LCMS study revealed that the distribution of several (57) bioactive compounds, such as (+/−)-3-[(2-methyl 3-furl) thiol]-2-butanone, 8-hydroxy-2-chlorodibenzofuran, sinapoyl malate, and other phytochemicals. It reveals that there was a considerable number (57) of metabolites present in the MEEL as only a few metabolites have been focused on in the present study.

The present study also revealed the pharmacological richness of *E. linneanum* wherein 30 bioactive chemical compounds were identified. Examples include steroid compounds like 23-acetoxysoladulcidine and Rhodexin A, flavonoids like 2″, 3″-Di-O-p-coumaroylafzelin, and alkaloids like pheophorbide a and pyropheophorbide a. Out of 57 identified phytoconstituents, 21 bioactive compounds had good oral bioavailability, and certain compounds were promising and

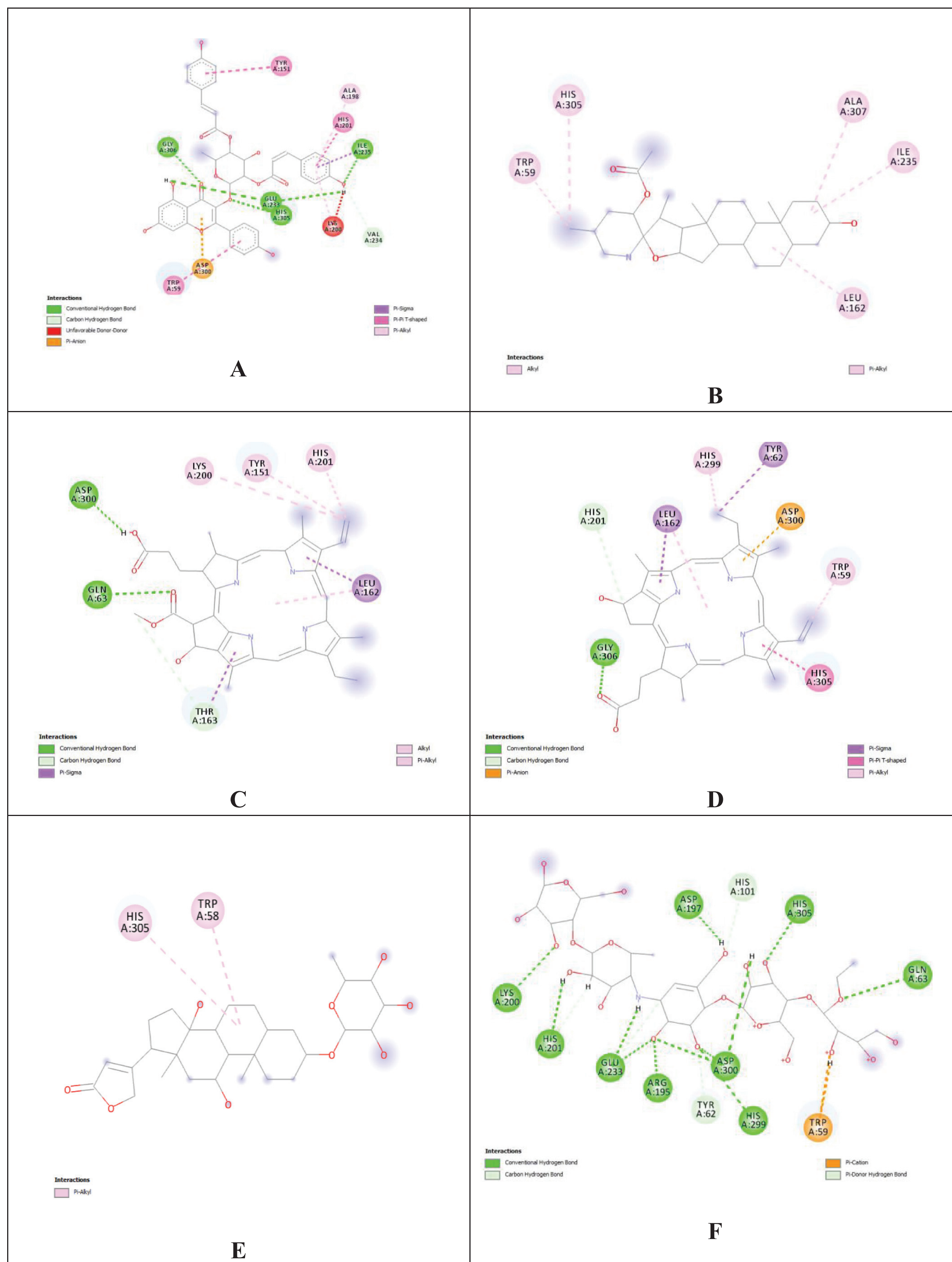

Figure 7. 2D interactions of phytochemicals and reference standard with best docking scores at active cites of 1b2y. (A = 2″,3″-Di-O-p-coumaroylafzelin; B = 23-Acetoxysoladulcidine; C = Pheophorbide a; D = Pyropheophorbide a; E = Rhodexin A; F = Acarbose).

Table 6. Binding energy of phytochemicals in docking study against 1b2y.

S. no.	Compound name	Binding energies (Kcal/Mol)
1.	1α-*O*-Methylquassin	−8.7
2.	2-(3-Phenylpropyl)tetrahydrofuran	−5.9
3.	2″,3″-Di-*O*-*p*-coumaroylafzelin	**−9.8**
4.	2,4,6-Trihydroxytoluene	−5.6
5.	2,7-Anhydro-α-*N*-acetylneuraminic acid	−6.8
6.	2′-Aminoacetophenone	−5.2
7.	4,4′-Diapo-zeta-carotene	−8.1
8.	5-(3-Hydroxy-4-acetoxybut-1-ynyl)-2,2′-bithiophene	−6.7
9.	5-Methoxytryptophol	−6.2
10.	7b-Hydroxy-3-oxo-5b-cholanoic acid	−9.1
11.	23-Acetoxysoladulcidine	**−9.6**
12.	C16-Sphinganine	−5.7
13.	D8′-Merulinic acid A	−6.4
14.	Fenapanil	−6.7
15.	Fraxidin	−6.4
16.	Isoscopoletin	−6.7
17.	Morindone	−8.4
18.	Nonyl octanoate	−5.1
19.	Perlolyrine	−8.1
20.	Pheophorbide a	**−9.5**
21.	Phytosphingosine	−5.9
22.	Pirbuterol	−6.9
23.	Procyanidin B7	−9.2
24.	Pyropheophorbide a	**−9.5**
25.	Retapamulin	−8.9
26.	Rhodexin A	**−9.6**
27.	Ricinine	−5.1
28.	Sinapoyl malate	−6.7
29.	Acarbose	−15.7

The binding energy shown in bold for best ligands.

Table 7. Binding interactions of phytochemicals at the active cite of 1b2y.

S. no.	Compound	Type of interaction		Interacting amino acid residues	Bond length (Å)
1	2″,3″-Di-O-*p*-coumaroyl afzelin	H-bond	Conventional	Glu233, Ile235 His305, and Gly306	2.84, 2.40, 1.88 and 2.53
			Carbon H-bond	Val234	3.62
			Donor-donor	Lys200	2.43
		Hydrophobic	Pi-anion	Asp300	4.33
			Pi-sigma	Ile235	3.43
			Pi-pi stacked	Trp59, Tyr151 His201	4.97, 5.21, and 4.49
			Pi-alkyl	Ala198	5.46
2	23-Acetoxy soladulcidine	Hydrophobic	Alkyl	Ile235, Ala307, Leu162	5.15, 5.27, and 5.39
			Pi-alkyl	Trp59, His305	4.39 and 4.79
3	Rhodexin A	Hydrophobic	Pi-alkyl	Trp58 and His305	5.22 and 5.47
4	Pheophorbide A	H-bond	Conventional	Gln63, Asp300	2.02, and 1.97
			Carbon H-bond	Thr163	3.57
		Hydrophobic	Pi-sigma	Leu162 and Thr163	3.77 and 3.68
			Pi-alkyl	Leu162 and Lys200, His201	5.21, 4.63 and 4.63
			Alkyl	Tyr151	4.99
5	Pyropheophorbide A	H-bond	Conventional	Gly306	2.58
			Carbon H-bond	His201	3.52
		Hydrophobic	Pi-anion	Asp300	4.50
			Pi-alkyl	Try59, Leu162 His299	3.59, 3.88, and 4.67
			Pi-sigma	Leu162, Tyr62	3.88 and 3.66
			Pi-pi-T-Stacked	His305	5.03
6	Acarbose	H-bond	Conventional	Trp59, Gln63, Arg195, Asp197, Lys200, His201, Glu233, His299, Asp300, His305	2.37–3.12
			Carbon H-bond	His101, Glu233, His201	2.46, 2.85, and 3.17
			Pi-donor H-bond	Tyr62	3.68
		Hydrophobic	Pi-cation	Trp59	3.42

supported Lipinski's rule with zero violations in an *in silico* SwissADME study. Around 28 phytoconstituents listed in Table 4 were selected and performed *in silico* prediction using acarbose as the standard.

***In silico* ADME study**

Online tools, such as the Swiss ADME free version, were used to evaluate drug-likeness and other pharmacokinetic parameters. A few compounds were found to have strictly adhered to Lipinski rule 5. Table 5 displayed the ADME profile including GI absorption, BBB permeability, and Lipinski rule violations.

***In silico* docking study**

Since α-amylase (PDB ID: 1b2y) is one of the key enzymes that facilitates the release of glucose into the bloodstream, it was selected as a target for *in silico* docking study. One of the potential approaches to regulating blood sugar involves inhibiting its enzyme activity. Thirty compounds were chosen based on their *m/z* values, and their properties were predicted *in silico* with acarbose as the reference compound. The *in silico* results demonstrated that the 23-acetoxysoladulcidine (−9.6 kcal/mol), rhodexin A (−9.6 kcal/mol), pheophorbide a (−9.5 kcal/mol), and pyropheophorbide a (−9.5 kcal/mol) were significant binding affinities against target (Fig. 7). The binding energies and active-site interactions that are crucial to their binding of ligands screened has been indicated in Tables 6 and 7.

It tends to be of utmost importance to use medicinal herbs as antioxidants and in the treatment of diabetes. This is said to be a result of the safety stigma linked to plants, the high cost, the abundance of adverse effects, and the lack of orthodox medications. The advantages of the secondary metabolites that these plants make to defend themselves from outside invaders are being highlighted in an increasing number of studies. MEEL is abundant in alkaloids, phenols, flavonoids, steroids, and tannins, according to a phytochemical study. For their capacity as antioxidants, tannins, phenols, and flavonoids are widely known. GC-MS and HR-LCMS analysis revealed about 67 phytoconstituents, in which few compounds (Ex. γ-sitosterol) have been found to be anti-diabetic activity [22]. Among these 21 compounds met the drug-likeness and other pharmacokinetic parameters when tested *in silico*. Only 6 of the 28 compounds tested by docking studies had good binding energy. According to the GC-MS and HR-LCMS metabolite profiling results, the volatile compounds γ-sitosterol, and pheophorbide A have been reported to be hypoglycemic.

CONCLUSION

The experimental investigations on PEEL, EAEL, and MEEL suggested that the leaves of the plant were enriched with several biologically active compounds. The MEEL contains a high concentration of flavonoids and phenols, which may account for its high antioxidant activity. *In vitro*, antidiabetic testing revealed that MEEL significantly inhibited α-amylase activity when compared to acarbose. It has also been reported for the first time on HR-LCMS-based identification of possible phytochemicals, and the profile showed 57 compounds, of which 21 compounds showed significant physiochemical and pharmacokinetic profiles. Among these 2″,3″-Di-O-p-coumaroylafzelin, 23-acetoxysoladulcidine, pheophorbide a, pyro pheophorbide a, and rhodexin, etc. The *in silico* analysis of the discovered phytochemical compounds predicted that they would have excellent bioavailability and good binding energies. The GC-MS analysis demonstrated the distribution of a few bioactive compounds. According to the literature, one of the compounds γ-Sitosterol has antihyperglycemic activity, and have significant antioxidant activity. Additional research is needed to isolate the more abundant compound in the MEEL by using different methods and to continue further studies for the identified compounds. In future isolation of responsible phytochemicals and their *in vivo* hypoglycemic and antioxidant assessment may lead to the development of effective novel natural agents.

ABBREVIATIONS

EAEL: Ethyl acetate extract of *E .linneanum*; FC: Folin ciocalteu; GC-MS: Gas chromatography-mass spectroscopy; HR-LCMS: High-resolution liquid chromatography-mass spectrometry; MEEL; Methanolic extract of *E. linneanum*; PEEL: Petroleum ether extract of *E. linneanum*; TPC: Total phenolic content; TFC: Total flavonoid content.

ACKNOWLEDGMENTS

The authors thank the authorities of KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India for providing the infrastructure to conduct the research.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FUNDING

There is no funding to report..

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Nallapaty S, Malothu N, Konidala SK, Areti AR. Evaluation of *in vitro* antidiabetic and antioxidant activity of leaf extracts of *Ecbolium linneanum* kurz.: GC-MS and HR-LCMS-based metabolite profiling and an *in silico* approach. J Appl Pharm Sci. 2024;14(01):247–260.